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Metabolism of Gibberellin A_{12}-7-Aldehyde by Soybean Cotyledons and Its Use in Identifying Gibberellin A_7 as an Endogenous Gibberellin[1]

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ABSTRACT

The level of gibberellin(GA)-like material in cotyledons of soybean (*Glycine max* L.) was highest at mid-pod fill—about 10 nanograms GA_3 equivalents per gram fresh weight of tissue, assayed in the immersion dwarf rice bioassay. This amount is about 1000-fold less than levels in *Pisum* and *Phaseolus* seed, other legume species whose spectrum of endogenous gibberellins (GAs) is well known. The metabolism of [^{14}C]-GA_{12}-7-aldehyde (GA_{12}ald)—the universal GA precursor—by intact, mid-pod-fill, soybean cotyledons and their cell-free extracts was investigated. In 4 hours, extracts converted GA_{12}ald to two products—[^{14}C]GA_{12} (42% yield) and [^{14}C]GA_{15} (7%). Within 5 minutes, intact embryos converted GA_{12}ald to [^{14}C]GA_{12} and [^{14}C]GA_{15} in 15% yield; 4 hour incubations afforded at least 22 products (96% total yield). The putative [^{14}C]GA_{12} was identified as a product of [^{14}C]GA_{12}ald metabolism on the basis of co-chromatography with authentic GA_{12} on a series of reversed and normal phase high pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC) systems, and by a dual feed of the putative [^{14}C]GA_{12} and authentic [^{14}C]GA_{12} to cotyledons of both peas and soybeans. The [^{14}C]GA_{15} was identified as a metabolite of [^{14}C]GA_{12}ald by capillary gas chromatography (GC)-mass-spectrometry-selected ion monitoring, GC-radiocounting, HPLC, and TLC. By adding the [^{14}C] metabolites of [^{14}C]GA_{12}ald to a different and larger extract (about 0.2 kg fresh weight of soybean reproductive tissue) and purifying endogenous substances co-chromatographing with these metabolites, at least two GA-like substances were obtained and one identified as GA_7 by GC-mass spectrometry. Since [^{14}C]GA_9 was not found as a [^{14}C]metabolite of [^{14}C] GA_{12}ald, soybean embryos might have a pathway for biosynthesis of active, C-19 gibberellins like that of the cucurbits; GA_{12}ald → GA_{12} → GA_{15} → GA_{24} → GA_{36} → GA_4 → GA_7.

Soybean (*Glycine max*) has been the subject of many studies on phytohormones, but few, if any, deal with endogenous GAs[3] or their metabolism. Identifying endogenous soybean GAs and elucidating the pathways for their synthesis is a first step in understanding the roles that GAs play in the physiology of this major crop. Since seeds of many legumes are relatively rich sources of GAs (*e.g.* up to 125 μg/g fresh weight in *Phaseolus coccineus* [8]), this suggests soybean seeds should be the first tissue examined for this purpose.

The GAs in extracts of higher plants usually have been prepared for identification by solvent partitioning, followed by either silica gel partition chromatography or reverse phase HPLC (5). After one or two such LC steps, fractions demonstrating biological activity, or pools of fractions with LC Rts similar to known GAs can usually be analyzed by GC-MS (*e.g.* 1). However, this approach has not yielded detectable levels of any GAs in immature seeds or seedlings of soybean (V Sponsel, personal communication to P Birnberg and M Brenner).

The failure of these traditional methods to identify any GAs in soybeans suggests either that soybean GA levels are lower than in most seeds, or that their presence is masked by compounds which are not removed by the minimal purification procedures used. Therefore, we decided to study soybean cotyledon metabolism of [^{14}C]GA_{12}ald—the universal, committed precursor of GAs (11)—because: (a) developing cotyledons have proved to be the most abundant source of GAs in other legumes (6, 7); (b) the radioactive products may allow identification of the GA pathways in soybeans; and (c) the [^{14}C] products could serve as markers for endogenous GAs during purification of large-scale extracts of soybean seeds.

In a test system, Maki *et al.* (19) found that [^{14}C]GA_{12}ald was metabolized by intact pea cotyledons primarily by the early-13-hydroxylation pathway,

$$[^{14}C]GA_{12}\text{ald} \rightarrow [^{14}C]GA_{53} \rightarrow [^{14}C]GA_{44} \rightarrow [^{14}C]GA_{19/17} \rightarrow [^{14}C]GA_{20}$$

the major GA pathway which had been suggested by enzymolog-

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[3] Abbreviations: GA, gibberellin; BSTFA, bis-trimethyl-silyl-trifloroacetamide; *n*-BuOH, 1-butanol; DAF, days after flowering; DMAP, *N,N*-dimethyl-amino pyridine; EGME, etiocholane-3α-ol-17-one glucuronide methyl ester; EtOAc, ethyl acetate; EtOH, 95% ethanol (aq); FID, flame ionization detector; GC-SIM, gas chromatography-selected ion monitoring; HOAc, acetic acid; iPrOH, 2-propanol; KA, ent-kaurenoic acid; KRI, Kovats Retention Index; LC, liquid chromatography; RC, radiocounting; Rt, retention time; Sys, system; amu, atomic mass units.

ical studies (13) and by identification of endogenous GAs (7, 9). These radioactive compounds were also used as markers to co-purify endogenous GAs from pea reproductive tissue, yielding, after three HPLC steps, >95% chemically pure $GA_{17/19}$, GA_{20}, GA_{44}, and GA_{53}, and providing the first identification of GA_{53} as an endogenous component of pea tissue. A similar procedure has been followed with soybeans and the results are presented herein.

MATERIALS AND METHODS

HPLC Systems. System 1a employed a RoSil C_{18} column 10 × 150 mm and a solvent flow rate of 4 ml/min. All other columns were 4.6 × 150 mm with solvent flow rates of 1 ml/min. Systems 1b, 2a-2g, and 6a-6c employed a Nucleosil 5 μm C_{18} column; system 3 a PRP-1 column; and systems 4 and 5a-5b a Chromosorb Silica column. All compounds were methylated with CH_2N_2 prior to separation on systems 4, 5, and 6. Systems 1a-1b, 3, and 4 employed the following linear solvent gradient programs—Systems 1a-1b with solvents (A) 0.1 M HOAc(aq), and (B) 0.1 M HOAc in CH_3CN: gradient from 100% to 80% A/20% B in 2 min, gradient to 35% B in 15 min, gradient to 75% B in 15 min, gradient to 100% B in 2 min, isocratic 100% B for 7 min; system 3 with solvents (C) 10 mM NaH_2PO_4 in 10% EtOH(aq) and (D) 1 mM Na_2HPO_4 in 75% EtOH9aq): gradient from 100% C to 100% D in 26 min, isocratic 100% D for 7 min; and system 4, isocratic 1% iPrOH/99% C_6H_{14} for 2 min, gradient to 25% iPrOH/75% C_6H_{14} in 24 min, isocratic 25% iPrOH/75% C_6H_{14} for 4 min. System 2a-2g, 5a-5b, and 6a-6c employed isocratic elution with 45% A/55% B (2a), 50% B (2b), 45% B (2c), 40% B (2d), 35% B (2e), 27% B (2f); 0.2% iPrOH/99.8% C_6H_{14} (5a), 1% iProH/99% C_6H_{14} (5b); 75% CH_3CN(aq) (6a), 58% CH3CN(aq) (6b), and 55% CH_3CN(aq) (6c).

Injection volumes were about 500 μl. Radioactive compounds eluting from the columns were detected by continuous, heterogeneous scintillation counting with a Packard Trace 7140; and organic material by UV absorbance at 205 nm (the detection limit for $GA_{4/7}$ was about 100 ng.)

TLC Systems. Silica gel plates were developed with toluene:EtOH:concn.NH_3(aq) (155:83:1, v/v) for system I; CH_2Cl_2:EtOAc: HOAc (70:30:1, v/v) for system IIa, 50:50:1 for system IIb, and 40:60:1 for system IIc; or hexane:iPrOH:HOAc (90:10:1) for system III.

Gas Chromatography-Mass Spectroscopy. GC-MS was performed with a Kratos MS-25 at 70 eV essentially as described (19). Prior to GC-MS, all GA had been methylated. They were trimethylsilated by incubation with 10 μl pyridine + 10 μl BSTFA + 5 μg DMAP at 22°C for 20 min. Ten-μl samples were injected into the GLC. KRI were estimated from parallel runs with 'Parafilm' dissolved in hexane (10). Recording of mass spectra was initiated 6 min after the GLC column reached 270°C. KRI of some standards were: Me-KA, 2457; Me-GA_9, 2500; MeTMS-GA_{20}, 2605; MeTMS-GA_4, 2614, MeTMS-GA_7, 2614; and MeTMS-GA_3, 2700. GC-SIM analysis of all samples except [^{14}C]GA_{15} was performed on this system.

Gas Chromatography-Selected Ion Monitoring of [^{14}C]GA_{15}. GC-SIM was performed essentially as described in Bottini *et al.* (3) with a final GLC temperature of 250°C.

Gas Chromatography-Radio Counting. Methylated GAs were treated with BSTFA and then subjected to GLC on an OV-101 column (2 mm × 183 cm) with a Hewlett Packard 5880A instrument. The carrier gas was He, flowing at 2.7 ml/min. The column was held at the injection temperature of 255°C for 10 min, linearly raised to 275°C in 2 min, and held at 275°C for 10 min. Chromatography on OV-17 was accomplished similarly with a 2 mm × 122 cm column eluted at 6 ml/min with a temperature program of 1 min at 158°C, 10 min linear increase to 300°C, and hold for 5 min. Retention times were determined by FID for all compounds except [^{14}C]D.1 and [^{14}C]D.2, and by RC (Barber Coleman Co., Series 5000) for [^{14}C]D.1, [^{14}C]D.2, and [^{14}C]GA_3. The delay between the FID and RC responses for the [^{14}C]GA_3 was used to calibrate the system, and all Rts adjusted accordingly.

Silica Partition Chromatography of Conjugates. The procedure of Koshioka *et al.* (17) was followed except that free GAs were eluted with EtOAc/HOAc (99/1), and column dimensions were 25 × 7 mm i.d. A model apolar conjugate, EGME, eluted in the methanol fraction and GA_8 in the EtOAc/HOAc fraction.

Plants. *Glycine max* (var Evans) seeds were planted in the field in St. Paul, MN on July 1, 1983 and July 5, 1984 in 76-cm rows, 3.8 cm apart, and plants were thinned to 7.6 cm 3 weeks later. Newly formed, white flowers on the fifth to seventh nodes were tagged 35 d after planting for future sampling. The pea plants used for studying [^{14}C]GA_{12} metabolism were grown and seeds harvested 22 DAF as described (19).

Isolation and Incubation of Reproductive Tissue with Radioactive Substrates. Soybean pods harvested 7 to 20 DAF were immediately stored at −80°C until extraction of the whole pods. Otherwise, cotyledons were isolated and stored at −80°C or treated with [^{14}C]GA_{12}ald or [^{14}C]GA_{12} as follows. A single cotyledon was placed on its abaxial surface upon moist filter paper and 5 μl of EtOH containing the [^{14}C]GA_{12}(ald) (about 50,000 dpm, 35 ng) was placed on the adaxial surface. After incubation in the dark at 22°C, cotyledons were frozen on dry ice and stored at −80°C until extraction.

Extraction and Isolation of GA. Tissues were extracted twice in 80% MeOH, and in the case of bulk extracts, partitioned against EtOAc as described (19). After acidic EtOAc partitioning, the aqueous phase of bulk extracts was partitioned against four volumes of *n*-BuOH.

Preparation of Radioactive Markers and Their Addition to Bulk Extracts. The radioactive products from a 4-h incubation of 24-DAF (about 50% filled) cotyledons with [^{14}C]GA_{12}ald consisted of a mixture of at least 20 [^{14}C]products, *e.g.* [^{14}C]B, [^{14}C]C, etc. These were added to bulk extracts, and the extracts then further purified. For purification of endogenous material co-eluting with [^{14}C]B and [^{14}C]D, 80-gm batches of 24-, 32-, 42-, and 52-DAF embryo tissue were pooled. For [^{14}C]E through [^{14}C]K, 30 gm of 7-to-20-DAF pods were pooled with 160 g of 24-DAF embryo tissue.

Purification of Bulk Extracts. The acidic EtOAc fractions of bulk extracts, mixed with the radioactive markers as described above, were concentrated *in vacuo* and subjected to HPLC system 1a with fractions collected every min. After aliquots were removed for bioassay, the 1-min fractions were pooled into peaks as indicated in Figure 1B. Each fraction was then separated on two or three more HPLC systems as follows: Fraction B—system 2b, then system 5a, then system 6a; D—2c, 5b, 6b; E—2a, 6c; F—2d, 3,4; G—2e, 3, 4; H—2e, 3, 4; J—2f, 3, 4; and K—2f, 3, 4. After each step, fractions co-eluting with the major [^{14}C] peaks were concentrated with an air stream and then lyophilized, except that after the last step, they were air-dried into 400-μl conical vials with iPrOH added to aqueous samples to hasten evaporation.

Bioassay of GA. The immersion, dwarf-rice (cv Tan-ginbozu) bioassay (23) was used. Quantifications were made by a standard curve which afforded a linear relationship between log (ng of GA_3) and log (length of second leaf sheath minus length of control [no GA]) over the range 1 to 100 ng; 1 ng GA_3 was barely detectable. Samples were assayed with one vial of five seedlings at each dilution.

Cell-Free Reactions. Cell-free extracts were prepared at 4°C by homogenizing cotyledons with 40 mM K-Hepes (pH 7.0), 0.6 M mannitol, 2.5 mM $MgCl_2$, 1 mM DTT, and 1 g/L BSA (1.6 ml buffer/g tissue). After centrifugation (12,000*g* × 30 min), three

volumes of the supernatant were incubated for 4 h at 22°C with one volume of reaction mix prepared to give the following final concentrations: NADPH, 1 mM; $MgSO_4$, 5 mM; $FeSO_4$, 0.5 mM; ascorbic acid, 5 mM; 2-oxoglutarate, 5 mM; [^{14}C]GA_{12}ald or [^{14}C]GA_{12}, about 0.2 nM; and pH 7.0. Reaction products were analyzed by HPLC system 1b.

Enzymic Hydrolysis of Conjugates. Presumptive conjugates were incubated for 0, 4, or 24 h at 22°C in 10 mM KP_i (pH 5.0) containing *A. niger* cellulase (3 mg/ml), *T. viridi* cellulase (3 mg/ml), almond β-glucosidase (3 mg/ml), and *A. niger* pectinase (1 mg/ml). This mixture is similar to Boots Pectinolysin (26).

Chemical Reactions. (a) Acetylation was accomplished by incubation overnight in pyridine/Ac_2O (2/1), and was monitored by TLC system III for $GA_{4/7}$ and by HPLC system 1b for [^{14}C]-D. (b) Saponification with NaOH was performed as described (13) and was monitored by HPLC system 1b. Methylation was performed with CH_2N_2 (19).

Materials. Co-factors and enzymes were purchased from Sigma. HPLC matrices were obtained from Alltech (RoSil C_{18}); Hamilton (PRP-1); Macherey-Nagel (Nucleosil C_{18}); or Merck (Spherisorb). EGME was synthesized by methylation of the corresponding acid (purchased from Sigma).

Authentic GA and KA were obtained or synthesized as follows: KA (24); $GA_{4/7}$ and GA_7 (>95%) from Abbott Laboratories; [^{14}C]GA_3 from Amersham; GA_{15} for GC-SIM from J. MacMillan; a mixture of GA_{15} and GA_9 for GLC-RC by isolation from *Thlaspi arvense* L. (20); [^{3}H]GA_8 and [^{3}H]GA_{20} (21); [^{3}H]GA_9 from A. Crozier (30). [2,3,5,6,9,11,13,14-^{14}C]GA_{12} (about 200 Ci/mol) and [2,3,5,6,9,11,13,14-^{14}C]GA_{12} (about 200 Ci/mol) were biosynthesized (2), as were [^{14}C]GA_{44} and [^{14}C]GA_{53} (19).

RESULTS

GA-Like Activity. To estimate the amount of GA-like activity in soybean cotyledons, extracts were made at five stages of development (Table I). The free GA-like substances were partitioned into acidic EtOAc and highly polar substances (most of the conjugated GAs) into acidic *n*-BuOH. The free GA were separated by HPLC system 1a into 30 fractions. Each HPLC fraction was assayed on Tan-ginbozu rice at two dilutions, 0.4 or 4 g fresh weight of tissue equivalents per vial. The *n*-BuOH fractions were each assayed without prior HPLC at 0.04, 0.4, and 4 g equivalents. No significant GA-like activity was detected in the *n*-BuOH fractions. The most active free GA preparation—from 24-DAF cotyledons—had many subfractions with GA-like activity (Fig. 1). Qualitatively similar patterns were obtained with the other preparations except for the 52-DAF extract, which had negligible bioactivity. GA-like bioactivity from soybean seeds was present in many fractions, but little was associated with fractions where GA_{19} or GA_{20} (the major bioactive 'early 13-hydroxy' GAs [7]) would have eluted (Fig. 1).

Total GA-like activity in all fractions (Table I) was highest at about the mid-pod-fill stage as in peas and runner beans, but was about 1000-fold lower (8, 9). The apparently low bioactivity does not reflect significant loss during sample purification since recovery of both polar and nonpolar [^{14}C]GAs added as internal standards was about 70% through this procedure; and since assaying mid-pod-fill peas in a similar manner gave the expected results (26). Endogenous inhibitor(s) of the immersion assay, probably ABA based on the Rt, may have obscured some GA bioactivity eluting with Rt 14-15 (Fig. 1D).

Table I. *Levels of Total GA-Like Substances in Soybean Reproductive Tissue at Five Stages of Development*

Days after Flowering	Percent of Maximum Seed Fresh Wt	Amt in GA_3 Equiv. (ng/g fresh wt)[a]
7–20	0–40	3[c]
24	50	3,[b] 10[c]
32	75	3[b]
42	100	2[b]
52	75[d]	0.3[b]

[a] Soybean cotyledons (24–52 DAF) or whole pods (7–20 DAF) were isolated from field-grown plants, extracted, and bioassayed on dwarf-rice as in Figure 1. [b] 1983 harvest. [c] 1984 harvest. [d] Dehydration phase.

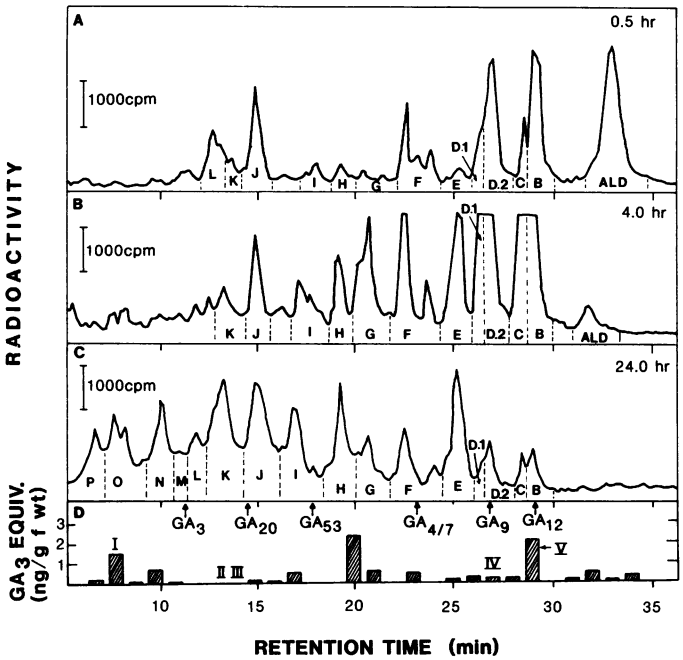


FIG. 1. A to C, Reversed-phase HPLC separation of the products of GA_{12}ald metabolism by half-filled (24-DAF) soybean cotyledons. Each cotyledon was incubated with [^{14}C]GA_{12}ald (about 40 ng, 200 Ci/mol) in the dark for 0.5, 4, or 24 h, extracted, and the products separated on HPLC system 1a. Each peak was assigned a letter from A to P. There was a slight malfunction of the HPLC pumps during run (B) causing the [^{14}C]GA_{12}ald to elute slightly early. D, Significant ($P < 0.05$) biological activity of an extract from equivalent soybean cotyledons in the immersion, dwarf-rice assay. The extract's acidic EtOAc fraction was separated as in (A–C), and 1 min fractions collected. Aliquots representing 4 g of tissue were assayed in duplicate. Fractions were also assayed in the presence of 10 ng GA_3; only for five fractions was the resulting activity less than expected. The apparent inhibition of the GA_3 exerted by these five fractions (indicated by roman numerals) were: (i) 50%; (ii) and (iii) 100% (total inhibition); (iv) 10%; and (v) 30%.

GA_{12}Aldehyde Metabolism. Preliminary experiments indicated that the metabolism of [^{14}C]GA_{12}ald was similar in 20, 24, 32, and 42 DAF cotyledons (40, 50, 75, and 100% filled, respectively). In a subsequent experiment with half-filled cotyledons, 0.5 h of metabolism afforded at least 12 [^{14}C]metabolites, and 4 h at least 20 (Fig. 1, A and B). After 24 h, the [^{14}C] products, most of which eluted prior to GA_{53} on C_{18} HPLC, were so numerous that the radiotrace response was well above baseline during the first half of the HPLC run (Fig. 1C).

When this experiment was repeated with metabolism terminated after shorter time intervals (5, 15, and 30 min), peak [^{14}C]B was the first product formed and peak [^{14}C]D.2 the second. Since, [^{14}C]B was postulated to be [^{14}C]GA_{12} (see below), the experiment was repeated with [^{14}C]GA_{12}. Most of the same [^{14}C] peaks were obtained, with [^{14}C]D.2 (later shown to be [^{14}C]GA_{15}) as the earliest major product.

The large number of [^{14}C] peaks in Figure 1, B and C suggested that some were GA conjugates. This hypothesis was tested in

two ways. First, aliquots of each peak [^{14}C]B to [^{14}C]O were individually separated on small, silica-partition columns into fractions which should contain free or conjugated GA (17). All the radioactivity associated with peaks N to O eluted in the putative conjugate fraction, as did about half of that from I, and about 20% of that from E. Second, each peak, [^{14}C]B to [^{14}C]K, was treated with a mixture of hydrolytic enzymes similar to Boots Pectinolysin (26) and the products analyzed on TLC system IIa, IIb, or IIc. Most of the radioactive substances in peaks E and I were converted to less polar products after 24 h (as determined by TLC), a result consistent with these peaks containing conjugated GA. The polarity of the radioactive compounds in the other peaks was unchanged by the enzymic treatment. Therefore, based on these two tests, even after 24 h of metabolism, only about one-quarter of the radioactive metabolites appear to be conjugated.

Cell-Free Metabolism. Since it has been reported (11) that cell-free preparations produce metabolites representative of endogenous GAs, the metabolism of [^{14}C]GA_{12}ald was investigated with crude enzyme extracts of cotyledons. Only three products were formed, but these products had the same HPLC system 1 Rt as [^{14}C]B and [^{14}C]D, and in one case, [^{14}C]G (chromatograms not shown). With extracts from 24 DAF cotyledons, a 4 h incubation afforded a 42% yield of [^{14}C]B, 7% of [^{14}C]D, and 1% of [^{14}C]G. Extracts of older cotyledons (32, 42, and 52 DAF) afforded less [^{14}C]B, and no [^{14}C]D or [^{14}C]G. With [^{14}C]GA_{12} as substrate, only [^{14}C]D was obtained—in 25% yield with extracts of 24 DAF extracts, and less with extracts of older tissues. Thus, soybean cotyledons contain enzymes capable of converting GA_{12}ald to 'B' (GA_{12}) and then 'D' (GA_{15}), with the younger tissues, which had more GA-like bioactivity than the older ones (Table I), having a greater capacity for converting GA_{12}ald to GA_{15}.

Identification of [^{14}C]B as [^{14}C]GA_{12}. Purification of [^{14}C]B on HPLC system 2b yielded a radiochemically pure substance (based on two TLC and three HPLC systems) with the Rt of [^{14}C]GA_{12} (system 2b, 10.6 min; system 5a, 9.0 min; and system 6a, 11.6 min). [^{14}C]B was immobile on TLC system I indicating that it was a dicarboxylic acid (28). Furthermore, on TLC systems IIa and IIb, the R_F of [^{14}C]B was slightly lower than that of GA_9 as would be expected for GA_{12} (14).

Only a single step is required to oxidize GA_{12}ald to the dicarboxylic acid GA_{12}, suggesting that [^{14}C]B was [^{14}C]GA_{12}. This hypothesis was confirmed by comparing the metabolism of [^{14}C]B to the metabolism of [^{14}C]GA_{12} in pairs of cotyledons isolated from the same embryo of both soybean and pea. In each case, the chromatographic patterns of the products of [^{14}C]B and [^{14}C]GA_{12} were nearly identical (Fig. 2).

Identification of [^{14}C]D.2 as [^{14}C]GA_{15}. Peak D was separated into two radioactive compounds by HPLC system 2c—a minor, component 'D.1' (Rt = 10.6 min), and 'D.2' (Rt = 12.0 min). We had an inadequate amount of authentic GA_{15} for use as an LC standard, but [^{14}C]D.2 co-chromatographed with GA_9 on TLC System I (R_F = 0.2), IIa (R_F = 0.8), and IIb (R_F = 0.95); and on HPLC system 2c and 6b (Rt = 11.4 min). GA_{15} and GA_9 have similar LC properties (16). The formation of GA_{15} from GA_{12} would require only one oxidation at C-20 followed by chemical esterification of GA_{15}-open lactone to GA_{15}. The formation of GA_9 from GA_{12} requires three steps [11]. [^{14}C]D.2 was unchanged by acetylation or saponficiation, consistent with the fact that GA_{15} has no free hydroxyl or ester groups (the lactone reforms on removal of the base [13]); model compounds GA_4 and GA_7 did react quantitatively with Ac_2O, and the model compound [^{14}C]GA_{44} was unchanged by saponification. We concluded that [^{14}C]D.2 was likely to be [^{14}C]GA_{15}.

The methyl esters of δ-lactone GAs have distinctively long Rt on GLC (5). [^{14}C]Me-D.2 and Me-GA_{15} co-eluted on two packed

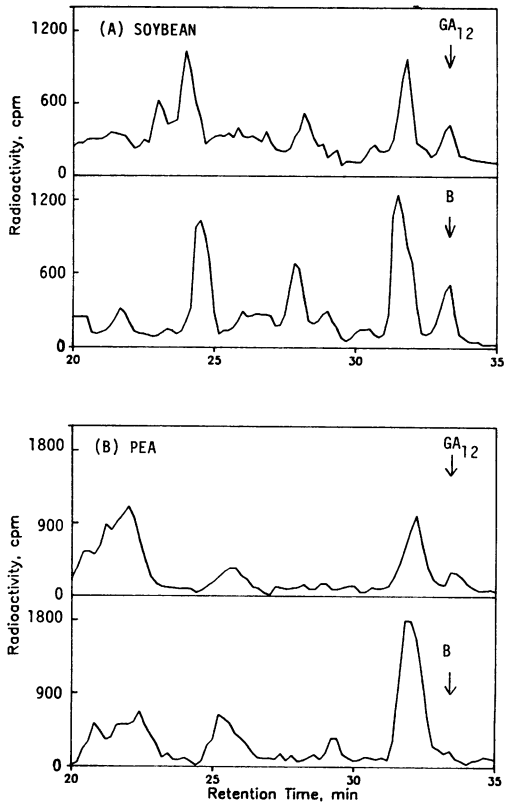


FIG. 2. Reverse-phase, C_{18} HPLC separation of the metabolites of [^{14}C]GA_{12} and [^{14}C]B incubated with (A) soybean or (B) pea cotyledons. Each of these two [^{14}C]compounds (about 15 ng) was incubated for 1 h with either cotyledon of a half-filled embryo, and the producets separated by HPLC system 1b. All [^{14}C] peaks eluted with Rts between 20 and 35 min. The arrow indicates the position of the starting compound—[^{14}C]-GA_{12} (upper graph) or [^{14}C]B (lower graph).

column, GLC-RC systems (10.6 min on OV-101, 1.8 min after GA_9 and only 0.1 min before GA_3; 13.4 min on the more polar OV-17, 3.2 min after Me-GA_3). Such Rts are very unusual for GAs as apolar as [^{14}C]Me-D.2 except for δ-lactones. On neither of these columns was any radioactivity associated with Me-GA_9 or any other FID peak except Me-GA_{15}. Based on its long Rt of 10.3 min on OV-101, [^{14}C]D.1 probably has a similar δ-lactone, and could be an artifact produced in isolating GA_{15}.

Additional evidence that [^{14}C]D.2 is [^{14}C]GA_{15} was provided by capillary-column GC-SIM. Authentic Me-GA_{15} eluted at 11.74 min (SD = 0.05 min) as indicated by the co-elution of its characteristic ions—344 m/e (M^+), 312, and 284. [^{14}C]Me-D.2 afforded co-eluting ions of 360, 328, and 300 amu at 11.68 min as would be expected for [2,3,5,6,9,11,13,14-^{14}C]MeGA_{15}, whose gibbane ring is 16 (8 × 2) amu heavier than that of [^{12}C]meGA_{15}. Peak D.2 also afforded the characteristic ions of [^{12}C]GA_{15} (m/e 344, 312, and 284), but it cannot be determined whether these are derived from endogenous GA_{15} or unlabeled GA_{15} produced from GA_{12}ald. Too little [^{14}C]Me-D.2 was available for the ratio of the characteristic ions to be meaningful, but in combination with the experiments described above, these GC-SIM data confirm that [^{14}C]Me-D.2 is [^{14}C]Me-GA_{15}.

Purification of Bulk Extracts and Identification of GA_7. The procedure (19) used to identify endogenous pea GAs was applied to soybean reproductive tissue. Markers of [^{14}C]B through [^{14}C]-K from a 4-h incubation of [^{14}C]GA_{12}ald (Fig. 1) were added to the acidic EtOAc fractions of bulk extracts of soybean reproductive tissue and the extracts purified through four HPLC systems (*cf.* "Materials and Methods"). During gradient C_{18} HPLC, ma-

terial co-eluting with each peak ([^{14}C]B to [^{14}C]K) was collected separately; during each subsequent HPLC step, material co-eluting with each of the major, distinct radioactive peaks was saved and subjected to further purification.

In contrast to the results with peas (19), there were no detectable UV-absorbing (205 nm) peaks with the same Rt as the radioactive markers. Even so, each fraction was analyzed for nonlabeled GAs by GC-MS, and by GC-SIM for characteristic ions of appropriate 'early 3-OH' or 'early 3,13-OH' GAs (5). In only two cases were mass spectra resembling those of known GAs obtained (Table II)—E.2 (which represented 34% of peak E after 4 h of metabolism) and F.2.2 (14% of peak F).

The spectrum afforded by E.2 (hereafter called 'GA_x'; HPLC Rts 5.4 min on system 2a and 8.8 min on system 6c) is not that of any known GA (5, 27) but has some fragmentation ions characteristic of Me-GA_9 (5). However, its GC Rt, almost as long as that of Me-TMS-GA_3, is several minutes more than either Me-GA_9 or 15,16-endorearranged Me-GA_9 (data not shown). Since its Rt on C_{18} HPLC was just less than that of GA_9, it may be a glucose conjugate of a GA similar to GA_9. However, no fragmentation ions characteristic of a TMS derivative of a sugar were detected (12).

The two GAs in fraction F.2.2 (HPLC Rts 21.2 on system 3 and 14.0 min on system 4) had mass spectra and KRI that matched GA_7 and iso-GA_7 (Table II). Iso-GA_7 was consistently obtained, albeit in varying amouts, during derivatization and GC-MS of GA_7 or $GA_{4/7}$. The possibility that the GA_7 may have been a contaminant was ruled out since the only form of GA_7 present in the laboratory during these experiments was $GA_{4/7}$, and no characteristic ions of GA_4 (418, 386, 284) were found in scans at or near KRI 2614, even though these two compounds will coelute through the purification scheme used.

Based on the height of the GLC-TLC trace and accounting for recovery during purification, the bulk extract of soybean cotyledons contained about 5 ng/g fresh weight of GA_7. Based on bioassay, peak F (HPLC fractions 22–24 in Fig. 1), contained about 0.5 ng/g fresh weight of GA_3-like bioactivity, equivalent to 1.5 ng/g fresh weight GA_7-like material in the immersion, dwarf-rice assay.

DISCUSSION

GA-like bioactivity in soybean cotyledons was much lower than in other legumes (*cf.* Table I) and more nearly approximated that found in seeds of rice (15). Unlike rice (15), GA-like bioactivity was also low in young soybean reproductive structures (Table I). The GA-like bioactivity in soybeans and the metabolites of [^{14}C]GA_{12}ald were present in many different HPLC fractions. Although she did not attempt to identify the metabolites, Nash (22) observed a similar large number of metabolites of [^{3}H]GA_{12}ald following its injection into young runner bean seedlings.

Soybean cotyledons have an active system for metabolizing GA_{12}ald. Within 5 min, intact cotyledons converted [^{14}C]-GA_{12}ald to [^{14}C]GA_{12} and [^{14}C]GA_{15} in 15% yield (data not shown), and within 4 h, ≥90% of the [^{14}C]GA_{12}ald was converted to at least 20 compounds (*cf.* Fig. 1). Although the half-filled soybean cotyledons had about 1000-fold lower levels of free GA-like substances than comparable pea tissue, the soybeans metabolized [^{14}C]GA_{12}ald at about one-eighth the efficiency of peas (compare Figs. 1 in [19] and herein). Similarly, cell-free preparations of soybean metabolized [^{14}C]GA_{12} or [^{14}C]GA_{12}ald at about one-fourth the efficiency of pea preparations—*e.g.* 25 and 49% oxidations of 0.2 μM GA_{12} and GA_{12}ald in 4 h for soybean; 87% conversion of 17.4 μM GA_{12} to GA_{53}; and 99% conversion of 2.4 μM GA_{12} to GA_{15} for pea under similar conditions (14). (Since these substrate concentrations are near the reported K_m values of GA precursors with microsomal oxidases (0.2–11 μM [11]), these conversion percentages probably are approximately proportional to enzyme titers). The relatively rapid metabolizing potential (at least for GA_{12}ald and GA_{12}) of soybean cotyledons and their cell-free extracts, coupled with the very low levels of endogenous free GA, may indicate a high rate of GA turnover in the reproductive tissues to inactive compounds.

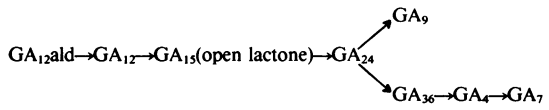
Since both intact cotyledons and their cell-free extracts converted [^{14}C]GA_{12}ald to [^{14}C]GA_{12} and then to [^{14}C]GA_{15}, this

Table II. *Mass Spectra of Gibberellin-Like Substances Isolated from Soybean Cotyledons*

Putative Gibberellin	Associated Metabolite of [^{14}C]GA_{12}ald	KRI	Mass Spectrum[a] m/e (% of base peak)
Me-TMS-GA_7	Me-TMS-F.2.2	2616	416 (M^+, 17) 384 (42) 356 (50) 333 (24) 312 (28) 311 (24) 298 (24) 270 (33) 269 (26) 244 (30) 243 (21) 223 (59) 222 (100) 221 (40)
Authentic Me-TMS-GA_7		2614	416 (M^+, 20) 384 (36) 356 (51) 312 (25) 311 (25) 298 (41) 281 (44) 270 (17) 269 (19) 244 (19) 242 (25) 223 (68) 222 (100) 221 (39)
Me-TMS-iso-GA_7	Me-TMS-F.2.2	2633	416 (M^+, 21) 384 (6) 356 (7) 326 (13) 313 (14) 299 (11) 298 (10) 282 (36) 281 (9) 267 (17) 244 (14) 238 (19) 223 (50) 222 (100)
Authentic Me-TMS-isoGA_7		2631	416 (M^+, 16) 384 (17) 356 (20) 326 (9) 313 (10) 312 (12) 299 (6) 298 (24) 282 (30) 281 (21) 267 (12) 223 (55) 222 (100) 221 (23)
'GA_x'	Me-TMS-E.2	2655	344 (44) 330 (76) 317 (32) 316 (55) 303 (47) 302 (72) 298 (36) 288 (44) 281 (20) 272 (40) 271 (60) 270 (100) 257 (27) 256 (24) 243 (99) 242 (39) 229 (43) 228 (42) 214 (39) 211 (32)

[a] Fourteen (20 for GA_x) most prominent ions over 199 amu. [b] The mass spectra of authentic GA_7 and isoGA_7 were obtained in a separate experiment several months prior to the analysis of F.2.2 and E.2. Their KRI were obtained at the same time as F.2.2 and E.2 by using a mixture of $GA_{4/7}$.

probably represents an endogenous pathway. This sequence has been reported to begin two types of pathways leading to active (non-2β-hydroxylated) C-19 GAs:



The steps in the upper pathway have been demonstrated enzymically or by identification of endogenous GA in several other species (11, 13, 25). All of the enzymes of the lower pathway except the last have been isolated from *Cucurbita maxima* (11), and work with *Sechium edule* indicates that *Sechium* possesses the entire lower pathway (1, 4, 18). *Cyathea australis* may have both pathways except for the dehydrogenation of GA_4 to GA_7; this fern probably further oxidized GA_4 and GA_9 to 11-OH and 12-OH GAs (29). Soybeans may use the pathway to GA_7 just described since: (a) In 4 h, cotyledons did not convert [^{14}C]-GA_{12}ald to any detectable [^{14}C]GA_9 (*cf.* GLC-RC results). (b) GA_7 was observed as an endogenous component of the reproductive tissue.

The procedure outlined by Maki *et al.* (19) allowed us to isolate GA_7 and the GA_x component with sufficient purity to obtain clean mass spectra by GC-MS. Purification using bioassay alone probably would have been insufficient to yield fractions pure enough to identify by GC-MS. In fact, the bioactivity associated with GA_7 (Fig. 1), though significant, represented only 5% of the total bioactivity and thus might have been thought unimportant.

Two sets of observations require some discussion. First, most of the [^{14}C]GA_{12}ald metabolites did not serve as markers for detectable levels of endogenous GAs. Since each soybean cotyledon contained only about 1 ng of GA_3-like material, it is likely that the 40 ng of [^{14}C]GA_{12}ald fed to each cotyledon was well above the endogenous level of GA_{12}ald and that some of the products were artifacts of substrate overloading. Although others may have been 'natural' metabolites, equivalent nonlabeled endogenous GAs were presumably present at levels too low to detect.

Second, two prominent GA-like bioactive peaks (Fig. 1) which were theoretically present in sufficient quantities for GC-MS identification remain unidentified. The less polar one (Rt = 29 min, Fig. 1) co-eluted with GA_{12} on HPLC system 1. Since subsequent purification of material co-chromatographing with [^{14}C]B ([^{14}C]GA_{12}) afforded no known GAs, this bioactive peak contained no detectable endogenous GA_{12}.

The more polar bioactive peak (Rt = 20–21 min, Fig. 1) is not a major product of exogenous GA_{12}ald since the four major [^{14}C]GA_{12}ald metabolites which eluted with approximately this Rt (Fig. 1) did not serve as markers for detectable endogenous GA. The bioactive substance with Rt = 20 to 21 min may lie on a biosynthetic pathway occurring at a site which exogenously applied GA_{12}ald does not reach. Alternatively, it could represent a trace amount of GA_{36} which is about as active as GA_7 in the dwarf-rice assay and which elutes just before GA_7 on C_{18} HPLC (16). Since a negligible amount of [^{14}C]GA_7 was detected, probably very little of its logical precursors, [^{14}C]GA_{36} and [^{14}C]GA_4, were present, and [^{14}C]GA_{36} could have been a minor, and therefore ignored, component of peaks [^{14}C]G or [^{14}C]H.

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